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ORIGINAL RESEARCH

Hypoxia-Induced Aquaporin-3 Changes Hepatocellular Carcinoma Cell Sensitivity to Sorafenib by Activating the PI3K/Akt Signaling Pathway

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Purpose: Hypoxia-induced changes are primarily activated in patients with hepatocellular carcinoma (HCC) and long-term sorafenib exposure, thereby reducing the sensitivity to the drug. Aquaporin-3 (AQP3), a member of the aquaporin family, is a hypoxia-induced substance that affects the chemosensitivity of non-hepatocellular tumors. However, its expression and role in the sensitivity of hypoxic HCC cells to sorafenib-induced apoptosis remain unclear. The purpose of this study was to detect changes in AQP3 expression in hypoxic HCC cells and to determine whether these changes alter the sensitivity of these cells to sorafenib.

Materials and Methods: HepG2 and HepG2 hypoxic cell models were established and AQP3 expression was detected using quantitative real-time polymerase chain reaction (qPCR) and Western blotting. Furthermore, the role of AQP3 in cell sensitivity to sorafenib was evaluated via flow cytometry, Western blotting, and a CCK-8 assay.

Results: The results of qPCR and Western blotting showed that AQP3 was overexpressed in the HepG2 and Huh7 hypoxic cell models. Furthermore, AQP3 protein levels were positively correlated with hypoxia-inducible factor-1α (HIF-1α) levels. Compared with cells transfected with lentivirus-GFP (Lv-GFP), hypoxic cells transfected with lentivirus-AQP3 (Lv-AQP3) were less sensitive to sorafenib-induced apoptosis. However, the sensitivity to the drug increased in cells transfected with lentivirus-AQP3RNAi (Lv-AQP3RNAi). Akt and Erk phosphorylation was enhanced in Lv-AQP3-transfected cells. Compared with UO126 (a Mek1/2 inhibitor), LY294002 (a PI3K inhibitor) attenuated the AQP3-induced insensitivity to sorafenib observed in hypoxic cells transfected with Lv-AQP3. Combined with LY294002-treated cells, hypoxic cells transfected with Lv-AQP3RNAi were more sensitive to sorafenib.

Conclusion: The study results show that AQP3 is a potential therapeutic target for improving the sensitivity of hypoxic HCC cells to sorafenib.

Keywords: AQP3, hypoxia, hypoxic HCC cells, hypoxia-inducible factor 1α, PI3K/Akt and Erk signaling pathways, sorafenib resistance

Introduction

Despite improvements in the treatment for hepatocellular carcinoma (HCC) over the past few decades, it remains one of the deadliest cancers in the world.[1,2] HCC is the fifth most common cancer worldwide and the third leading cause of cancer death.[2] The prognosis of HCC is poor, most of which are in advanced stage. There are no reliable treatment methods for advanced stage.,[2,3] and the existing systemic treatment is limited by drug

resistance.[2,4] New treatment focus is needed to improve patient prognosis. The increased HCC sensitivity to targeted therapy suggests that the prognosis of patients with advanced HCC can be improved.[5–7]

Sorafenib is an oral multikinase inhibitor that blocks the RAF signaling pathway as well as inhibits vascular endothelial growth factor (VEGF), platelet-derived growth factor, and KIT expressions. Sorafenib exerts anti-proliferative and anti-angiogenic effects; however, its mechanism of action remains unclear.[2] It is the first and only promising targeted therapy for HCC and is currently the treatment of choice for patients with advanced disease.[8–11] However, HCC's long-term response to treatment is unsatisfactory, suggesting the presence of acquired resistance.[8,12-14] Extensive cumulative data from pre-clinical and clinical studies indicate that long-term sorafenib use, among other factors, aggravates tumor hypoxia, which is the main driving factor of the resistance against this drug.[3,15,16] During severe hypoxia, certain survival changes associated with tumor progression, metastasis, and drug resistance are activated;[16–18] however, to date, the complex mechanism underlying these changes remains unclear. Therefore, an improved understanding of this phenomenon is crucial to ameliorate the HCC sensitivity to sorafenib.

AQP3 is one of the aquaglyceroporins, which a[illegible] a subclass of aquaporins that afford permeability to wate[illegible] and glycerol.[19] Although all aquaglyceroporins (A[illegible]3, 7, 9, and 10) are expressed in the human liver, the AQP3 subtype is overexpressed mostly in HCC.[20–23] AQP3 role i[illegible] progression, prognosis, and treatment has recen[illegible] garnered attention. In addition to promotin[illegible]mor progres[illegible]n and metastasis,[24–28] AQP3 is involved in drug [illegible]istance in various malignant tumors.[29,30] AQP3 knockdown or [illegible]ckout studies have demonstrated its [illegible]e in v[illegible]ious tumor types.[29,31–33] Although the exact me[illegible]ani[illegible]m is unclear, AQP3 is an hypoxia-induced [illegible]nce a[illegible] has b[illegible]n linked to hypoxia-induced tu[illegible]or pr[illegible]gression, metastasis, and drug resistance.[2[illegible],31] St[illegible] shown that AQP3 expression is altered in hyp[illegible]c HCC cells; however, its effect on sorafenib sensitivity is un[illegible]ar.

We aimed to detect changes in AQP3 expression in hypoxic HCC cells and to determine whether these changes alter hypoxic cell sensitivity to sorafenib.

Materials and Methods

Cell Culture, Reagents, and Antibodies

Human HCC cell lines Huh7 and HepG2 were purchased (Chinese Academy of Sciences, Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (Gibco, Shanghai, China) and 1% penicillin/streptomycin and maintained in an incubator with a humidified atmosphere of 5% CO_2 at 37°C for the normoxic cell model or 1% O_2, 5% CO_2 at 37°C for the hypoxic cell model. Sorafenib, UO126 and LY294002 (Sigma-Aldrich, St. Louis, Missouri, USA) were purchased and dissolved in 100% DMSO to prepare a 10mM stock solution, which was then diluted with DMEM to the desired concentration, with a final concentration of 0.1% DMSO [illegible]commended for in vitro studies. Unless otherwise st[illegible]ed, all an[illegible]odies were obtained from Life Span BioSc[illegible]nces (LSBI[illegible], Seattle, Washington, USA). The enha[illegible]ed chem[illegible]lumines[illegible]nce detection kit was purchased f[illegible]m Amersham [illegible]acia Biotech (APB, Buckinghamshi[illegible] UK). The primers for AQP3 and GAPDH were o[illegible]tained f[illegible]m Sang[illegible] Biotech (Sangon Biotech, Shan[illegible] China). S[illegible] Premix Ex Taq™ (Tli RNaseH Plus) was p[illegible]chased from Takara Bio Inc (Takara, Beijin[illegible]a).

Cytotoxicity Assay

The di[illegible]rence i[illegible] the sensitivity toward sorafenib treatment [illegible] examined using a cell counting kit-8 assay (CCK-8), as p[illegible] the [illegible]gma-Aldrich (Sigma-Aldrich, St. Louis, Missouri, USA) CCK-8 protocol. Briefly, Huh7 and HepG2 cells were [illegible]eeded in a 96-well plate at a density of 5000 cells/well and incubated for 48 h under normoxic or hypoxic conditions, followed by replacement of the medium with culture medium with the indicated sorafenib concentration (0–24μM). After 24 h, 10μL of CCK-8 solution was added to each well of the plate, followed by 2 h incubation before detecting absorbance at 450nm using a microplate reader. Cell viability (as a percentage) was determined in relation to the average absorbance of the untreated cells from three replicate samples.

Annexin V Apoptosis Assay

The differences in apoptosis were examined using an Annexin V apoptosis assay. Briefly, Huh7 and HepG2 cells were seeded in 6-well plates at 2×10^5 per well for 48 h under normoxic or hypoxic conditions prior to treatment. After sorafenib treatment/transfection, cells were harvested via trypsinization, washed twice with Phosphate buffered saline (PBS), and stained with a fluorescein isothiocyanate Annexin V Apoptosis Detection kit (BD Pharmingen, Franklin Lakes, NJ, USA). Flow cytometry (Thermo Fisher Scientific,

Waltham, Massachusetts, USA) was used to determine the cell apoptosis ratio.

Protein Extraction and Western Blotting

Protein was extracted from HCC cell lines using RIPA lysis buffer as per the protocol (Pierce Biotechnology Inc, Rockford, Illinois, USA). Protein concentration was determined via the bicinchoninic acid protein assay as per the Bio-Protocol (bio-protocol, Philadelphia, Pennsylvania, USA). An equivalent of 30μg of the protein extract was resolved via sodium dodecyl sulfate polyacrylamide gel electrophoresis and electro transferred (wet) to polyvinylidene difluoride membranes (EMD Millipore Corporation, Billerica, Massachusetts, USA). The membranes were initially blocked with 5% BSA in TBST (137mM NaCl, 20mM Tris HCl [pH 7.6], and 0.1% [v/v] Tween 20) for 1 h, followed by incubation overnight at 4°C with primary antibodies (1:1000) against AQP3, Erk, phospho-Erk, Akt, phospho-Akt, HIF-1α, LC3B, p62, cleaved caspase-3, and GAPDH/beta-tubulin (as loading controls) and horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000). Immunodetection was performed using an ECL Western Blotting Detection Kit (Beyotime, Shanghai, China). The relative protein expression levels were quantified by densitometric measurement of ECL reaction bands and normalized to GAPDH/beta-tubulin levels.

RNA Extraction, Reverse Transcription, and qPCR

Total RNA was extracted from HCC cell lines using an RNAIsoPlus assay kit (Takara, Dalian, China) according to the manufacturer's instructions. After quantification, RNA was transcribed into cDNA using a two-step reverse transcription kit (Takara, Dalian, China). Subsequently, qPCR was used to detect target gene expression levels using the SYBR-Green qPCR master mix (Takara, Dalian, China). The thermocycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles of a two-step PCR of 95°C for 14 s and 60°C for 1 min. The $2^{-\Delta\Delta Cq}$ method was used to quantify the results; the relative expression level of AQP3 was normalized to that of GAPDH. The primers used were as follows: AQP3, forward (F), 5′–GGCTGTATTATGATGCAATCT–3′ and reverse (R), 5′–ATATCCAAGTGTCCAGAGG–3′. GAPDH F, 5′–GATCATCAGCAATGCCTCCT–3′ and R, 5′–GAGTCCTTCCACGATACCAA–3′. The data have been deposited in a publicly accessible database (GenBank) with accession number NM_004925.5 and NM_002046.7 for AQP3 and GAPDH, respectively.

Cell Transfection

Huh7 and HepG2 cells were plated in 6-well plates until 60% confluence and then infected with lentivirus-AQP3 (Lv-AQP3), lentivirus-AQP3RNAi (Lv-AQP3RNAi), or lentivirus-GFP (Lv-GFP)-control (GenePharma Co. Ltd, Shanghai, China) according to infection value multiplicity. The medium was replaced 14 h later and the expression of the GFP gene observed under a fluorescence microscope. The selection of cells with stable virus integration into the genome was performed by replacing the medium with a fresh medium containing 2μg/mL puromycin at 48 h posttransduction, followed by incubation for 10 days. The effect of transfection on AQP3 expression was assessed via qPCR and Western blotting.

Statistical Analysis

Statistical analyses were performed using SPSS 21.0. Parametric data are presented as the mean±standard deviation (SD). Between-group differences were analyzed using Student's *t*-test. Significance was set at $P < 0.05$(*), < 0.01(**), or < 0.001(***).

Results

Hypoxia Reduces Sensitivity to Sorafenib and Upregulates AQP3 Expression in HCC Cells

Here, we tested the hypothesis that the expression of AQP3 is upregulated in hypoxic HCC cells.

To test the hypothesis, the hypoxic cell model of the Huh7 and HepG2 cells were established based on previous studies[17] and HIF-1α expression verified via Western blotting. The changes of IC50 and apoptosis after sorafenib treatment were detected by CCK-8 and flow cytometry, respectively. CCK-8 assay results showed a dose-dependent inhibitory effect of sorafenib on normoxic and hypoxic cell activities; however, the IC50 value of hypoxic cells was significantly higher than that of the normoxic ones (Figure 1A). Flow cytometry showed a significantly lower apoptosis rate in the hypoxic group than in the normoxic group (Figure 1B). These results showed that hypoxia resulted in a decreased HCC cells sensitivity to sorafenib. Conversely, changes in AQP3 mRNA and protein levels in cell models were detected via qPCR and Western blotting, respectively. PCR results showed that the gene expression of AQP3 was significantly higher in the hypoxic cell models than in their normoxic counterparts (Figure 1C). Western blotting showed a similar trend (Figure 1D); additionally, it showed a significant positive

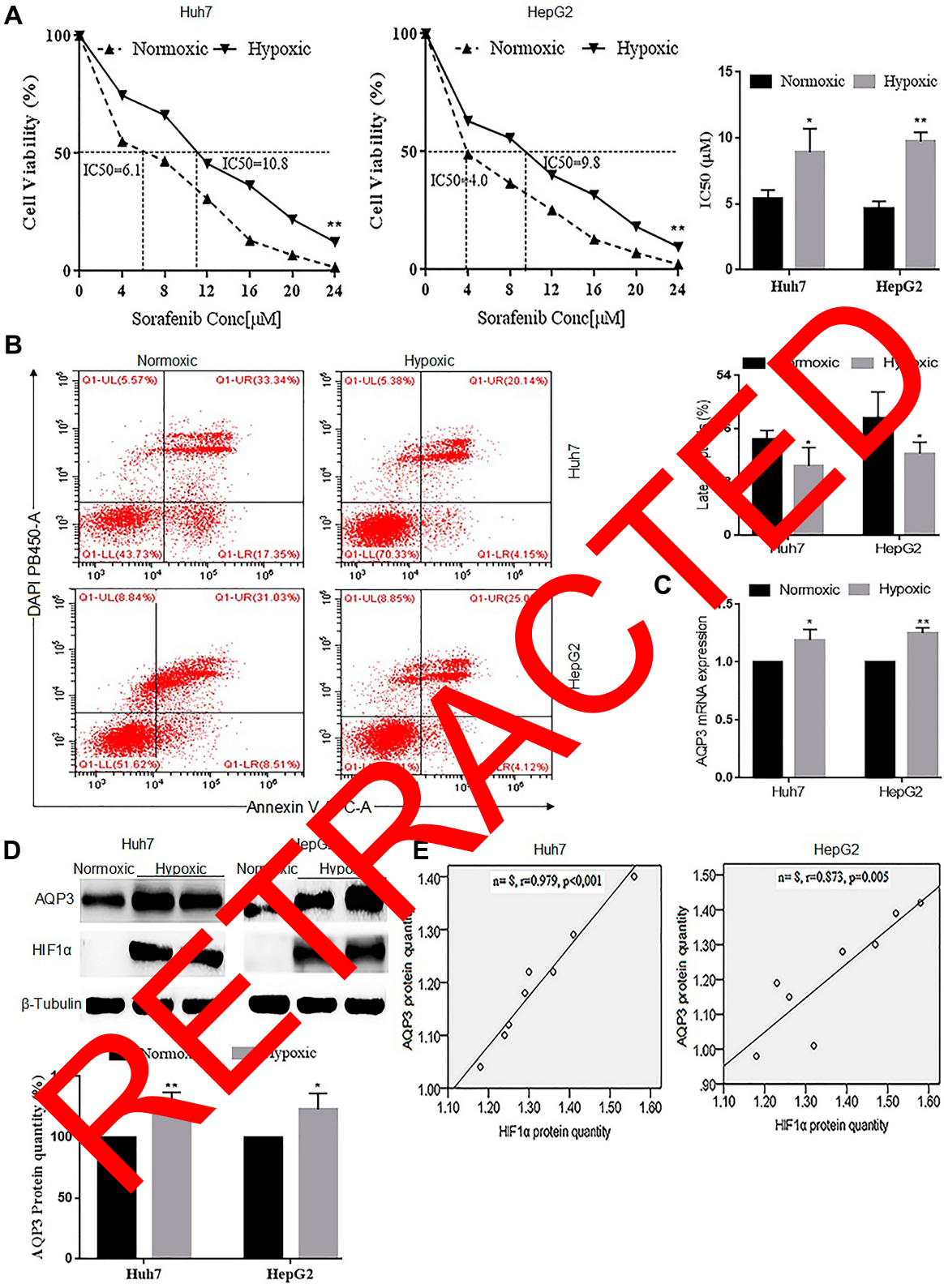

Figure 1 Hypoxia reduces the sensitivity to sorafenib and upregulates the expression of aquaporin-3 (AQP3) in hepatocellular carcinoma (HCC) cells. (**A**) IC50 values of sorafenib in hypoxic and normoxic Huh7 and HepG2 cells, as determined by the CCK-8 assay. (**B**) Proportion of apoptotic Huh7 and HepG2 cells in normoxic and hypoxic conditions after incubation with 6.8μM sorafenib for 24 h, as determined by flow cytometry. (**C**) Real-time polymerase chain reaction results showing the normalized AQP3 mRNA expression in normoxic and hypoxic Huh7 and HepG2 cells. (**D**) Western blotting results showing AQP3 protein levels relative to β-tubulin in normoxic and hypoxic Huh7 and HepG2 cells. (**E**) Pearson's correlation line graph of AQP3 and HIF-1α protein levels in hypoxic Huh7 and HepG2 cells. Each experiment was repeated four times, and the mean (SD) is shown in the histogram. Compared with the normoxic cell model (control), the differences were significant as follows: $P < 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$.

correlation between AQP3 and Hif-1α levels in hypoxic cells (Figure 1E). These results show that changes in AQP3 expression are potentially involved in the reduced hypoxic HCC cell sensitivity to sorafenib.

Hypoxia Reduces HCC Cell Sensitivity via PI3K/Akt Signaling Pathway Activation

Considering that the Erk and PI3K/Akt signaling pathways are involved in normal liver cancer cells' resistance to sorafenib[34,35] and that hypoxia induces Erk and PI3K/Akt activation in cells[17] here, we determined whether PI3K/Akt or Erk signaling pathway inhibitors could restore this sensitivity. Hypoxic cells were co-treated with 10μM U0126 (Mek1/2 inhibitor)[36] or 50μM LY294002 (PI3K inhibitor)[37] for 24 h. Subsequently, changes in protein levels, IC50, and apoptotic cell numbers were detected via Western blotting, CCK-8 assay, and flow cytometry, respectively. Western blotting results showed that hypoxia promoted Akt and Erk phosphorylation, suggesting PI3K/Akt and Erk signaling pathway activation in hypoxic HCC cells. This activation was successfully suppressed by LY294002 but not by UO126 (Figure 2A). CCK-8 assay results revealed that LY294002 significantly reduced the IC50 of sorafenib in the cells compared with UO126 (Figure 2B). Flow cytometry results demonstrated that co-treatment with LY294002 attenuated hypoxia-induces insensitivity to sorafenib in Huh7 and HepG2 cells (Figure 2C). These results suggest that the PI3K/Akt signaling pathway is involved in reducing hypoxic HCC cell sensitivity to sorafenib.

Hypoxia Upregulates AQP3 Expression in HCC Cells Through the PI3K/Akt Signaling Pathway

Based on our previous experiment findings, we hypothesized that hypoxia upregulates AQP3 expression through the Erk

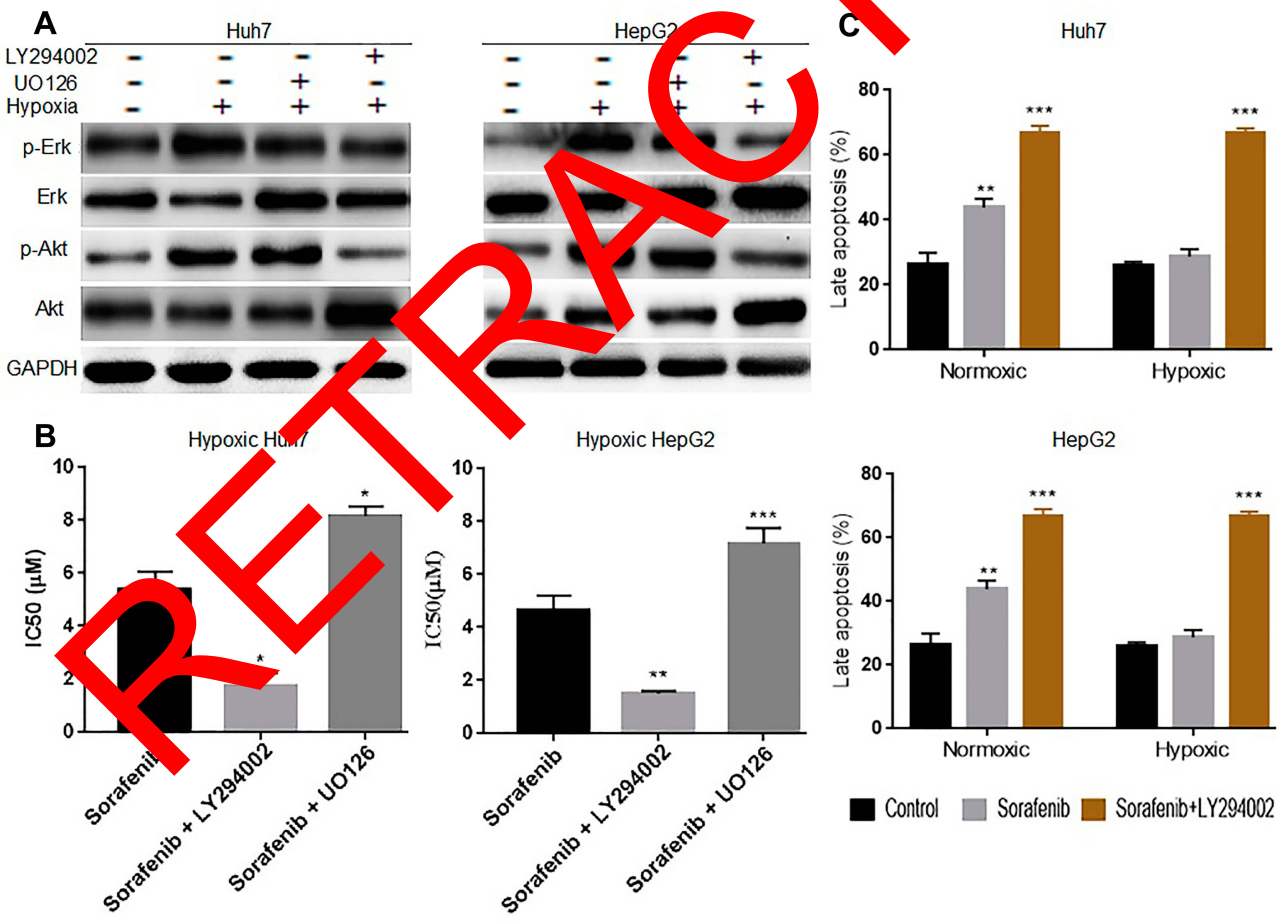

Figure 2 Hypoxia reduces hepatocellular cell sensitivity by activating the PI3K/Akt signaling pathway. (**A**) Western blotting showing that UO126 and LY294002 inhibited Erk and PI3K/Akt signaling, respectively, in hypoxic Huh7 and HepG2 cells. (**B**) CCK-8 results showing that the IC50 of hypoxic Huh7 and HepG2 cells was decreased in the sorafenib + LY294002 group, whereas it was increased in the sorafenib + UO126 group after 24 h of treatment. (**C**) The results of flow cytometry showed that the apoptosis rate was increased significantly in the sorafenib + LY294002 group, regardless of condition, in Huh7 and HepG2 cells after 24 h of treatment. Each experiment was repeated four times, and the mean (SD) is shown in the histogram. Compared with the control group (sorafenib alone/ctl), the differences were significant as follows: $P < 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

and PI3K/Akt signaling pathways. To validate this hypothesis, we examined whether PI3K/Akt or Erk signaling pathway inhibitors could attenuate the hypoxia-induced AQP3 upregulation in HCC cells. Western blotting was used to detect the protein levels of AQP3, p-Akt, Akt, p-Erk, and Erk in Huh7 and HepG2 hypoxic cells treated with 10μM U0126[37] or 50μM LY294002[37] for 24 h. The results showed lower AQP3 protein levels in LY294002-treated cells than in UO126-treated or untreated cells (Figure 3A). The increase in the p-Akt/Akt ratio was similar to that in AQP3, suggesting that PI3K/Akt signaling pathway activation causes AQP3 upregulation in hypoxic HCC cells (Figure 3B). These findings suggest that the PI3K/Akt signaling pathway is involved in AQP3 upregulation in hypoxic HCC cells.

AQP3 Upregulation Renders Hypoxic HCC Cells Insensitive to Sorafenib

We further investigated whether AQP3 upregulation affects hypoxic HCC cell sensitivity to sorafenib. Huh7 and HepG2 cells were transfected with Lv-AQP3, Lv-AQP3RNAi or Lv-GFP (control), and the transfection efficiency was verified via qPCR (Figure 4A and C) and Western blotting (Figure 4B and D). The transfected cells were exposed to hypoxia for 48 h and then incubated with sorafenib at different concentrations (0–24μM) for 24 h. IC50 and apoptosis rate were detected via CCK-8 assay and flow cytometry, respectively. In either case, the IC50 of Lv-AQP3-transfected cells was significantly increased, whereas that of Lv-AQP3RNAi-transfected cells was significantly decreased (Figure 4E). Consistent with CCK-8 assay results, flow cytometry results showed that apoptotic cell proportion was decreased in the Lv-AQP3-transfected group, whereas it was increased in the Lv-AQP3RNAi-transfected group (Figure 4F). These results suggest that AQP3 upregulation reduces hypoxic HCC cell sensitivity to sorafenib.

AQP3 Is an Upstream PI3K/Akt and Erk Signaling Pathway Regulator in HCC Cells

We investigated the effect of AQP3 expression on PI3K/Akt and Erk signaling pathway activation. Using Western blotting, p-Akt, Akt, p-Erk, and Erk protein levels were detected in Huh7 and HepG2 hypoxic cells transfected with Lv-AQP3, Lv-AQP3RNAi, or Lv-GFP (control). Western blotting was used to detect the activation of signaling pathways in Huh7 and HepG2 hypoxic cells transfected with Lv-AQP3,

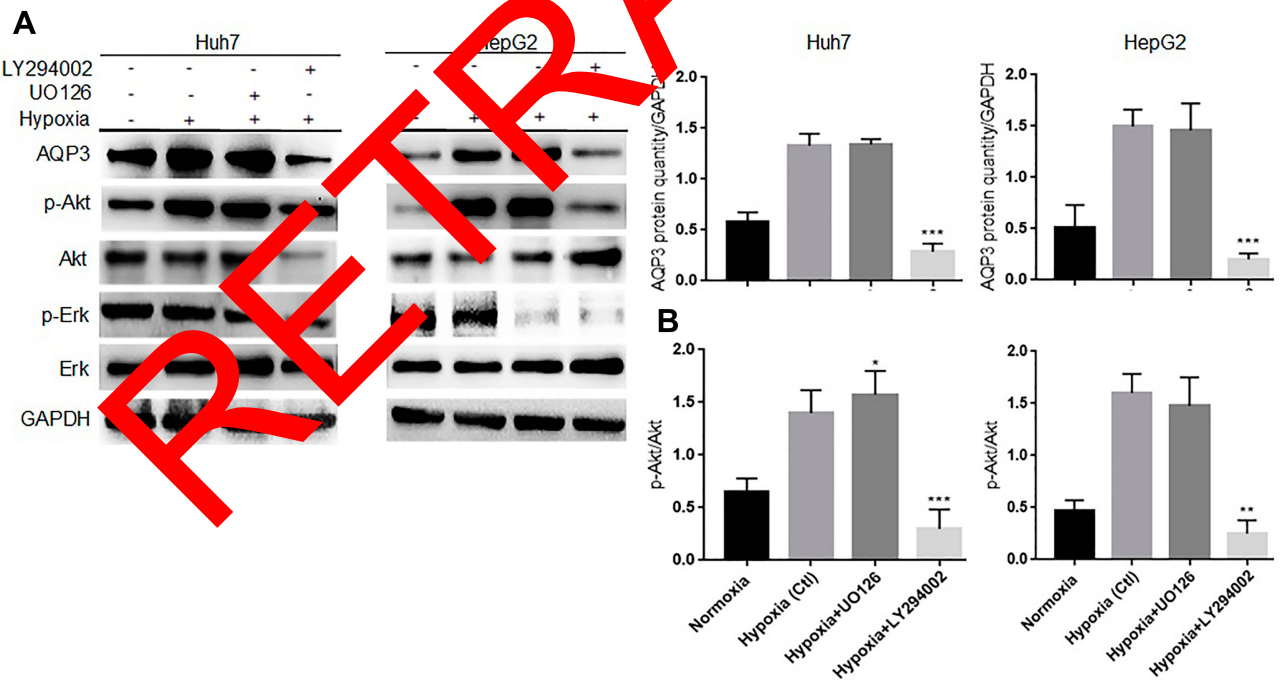

Figure 3 Hypoxia upregulates aquaporin-3 (AQP3) expression in hepatocellular carcinoma cells through the PI3K/Akt signaling pathway. (**A**) Western blotting showing that LY294002 successfully inhibited the PI3K/Akt and Erk signaling pathways and downregulated AQP3 in Huh7 and HepG2 hypoxic cells at 24 h after treatment. (**B**) Western blotting showing that the p-Akt/Akt ratio increased in the hypoxia or hypoxia + UO126 group, whereas it decreased in the hypoxia + LY294002 group. Each experiment was repeated four times, and the mean (SD) is shown in the histogram. Compared with the hypoxia group (Ctl), the differences were significant as follows: $P < 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

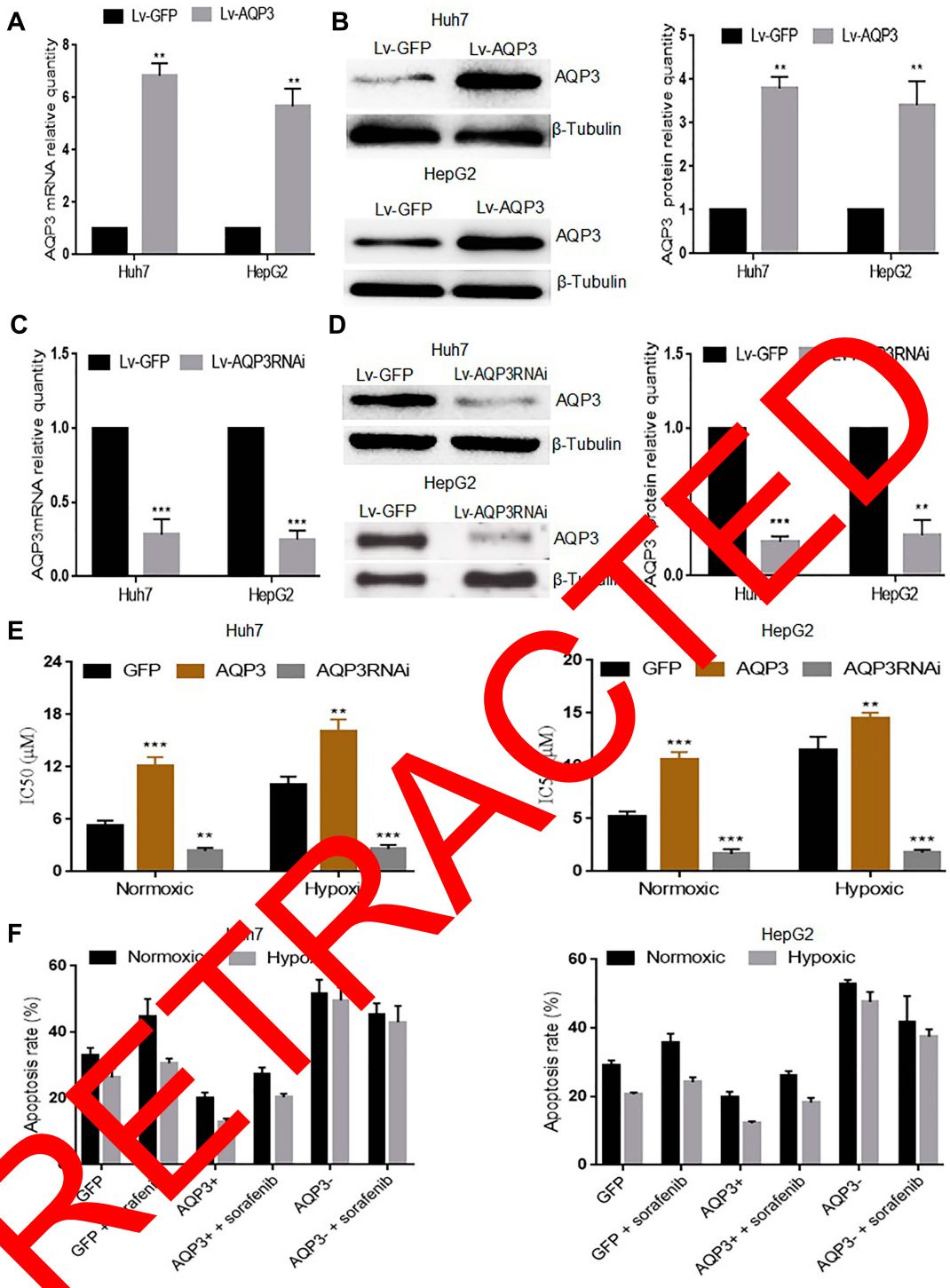

Figure 4 Aquaporin-3 (AQP3) overexpression renders hypoxic hepatocellular carcinoma cells insensitive to sorafenib. (**A**) Real-time polymerase chain reaction (qPCR) results showing the relative quantity of the AQP3 mRNA in Huh7 and HepG2 cells transfected with either Lv-AQP3 (AQP3+) or GFP (Ctl). (**B**) Western blotting results showing the relative quantity of the AQP3 protein in Huh7 and HepG2 cells transfected with either Lv-AQP3 (AQP3+) or GFP (Ctl). (**C**) qPCR results showing the relative quantity of the AQP3 mRNA in Huh7 and HepG2 cells transfected with either Lv-AQP3RNAi (AQP3−) or GFP (Ctl). (**D**) Western blotting results showing the relative quantity of the AQP3 protein in Huh7 and HepG2 cells transfected with either Lv-AQP3RNAi (AQP3−) or GFP (Ctl)). (**E**) CCK-8 assay results showing the IC50 of sorafenib in Huh7 and HepG2 cells transfected with either Lv-AQP3 (AQP3+), Lv-AQP3RNAi (AQP3−), or GFP (Ctl) both under normoxic and hypoxic conditions. (**F**) Flow cytometry results showing the proportion of apoptotic Huh7 and HepG2 cells transfected with either Lv-AQP3 (AQP3+), Lv-AQP3RNAi (AQP3−), or GFP (Ctl) after 24 h treatment with 6.8μM sorafenib. Each experiment was repeated four times, and the mean (SD) is shown in the histogram. Compared with the GFP group (Ctl), the differences were significant as follows: $P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.

Lv-AQP3RNAi, or Lv-GFP (control). The results showed that the signaling pathways were activated after transfection with Lv-AQP3 and inactivated after transfection with Lv-AQP3RNAi (Figure 5A and C). Autophagy is a downstream effector of the PI3K/Akt and Erk signaling pathways. LC3B-I conversion into LC3B-II and p62 marker accumulation indicated that autophagy was inhibited in the Lv-AQP3-transfected group and activated in the Lv-AQP3RNAi group (Figure 5B and D). These results suggest that AQP3 is an upstream PI3K/Akt and Erk signaling pathway regulator.

AQP3 Upregulation Renders Hypoxic HCC Cells Less Sensitive to Sorafenib by Activating the PI3K/Akt Signaling Pathway

Based on our previous experiment results, we hypothesized that AQP3 upregulation reduces hypoxic HCC cell sensitivity

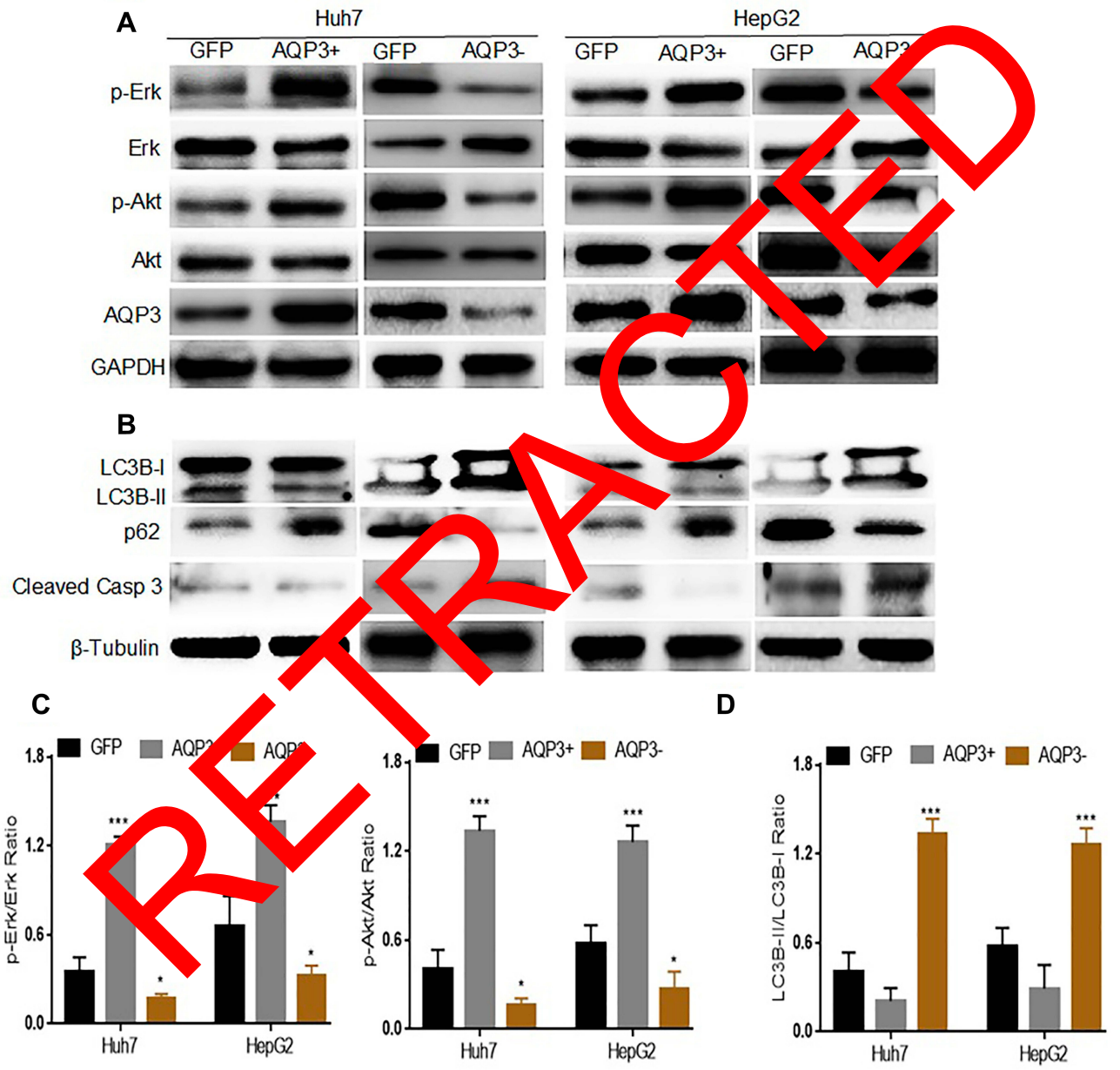

Figure 5 Aquaporin-3 (AQP3) is an upstream regulator of the PI3K/Akt and Erk signaling pathways in hepatocellular carcinoma cells. (**A**) Western blotting results showing the changes of p-Erk, Erk, p-Akt, Akt, and AQP3 protein levels in Huh7 and HepG2 hypoxic cells transfected with either Lv-GFP (Ctl), Lv-AQP3 (AQP3+), or Lv-AQP3RNAi (AQP3−). (**B**) Western blotting results showing the levels of cleaved caspase-3 and autophagy-related proteins, such as p62 and LC3B-II, in Huh7 and HepG2 hypoxic cells transfected with either Lv-GFP (Ctl), Lv-AQP3 (AQP3+), or Lv-AQP3RNAi (AQP3−). (**C**) Western blotting results showing the p-Erk/Erk and p-Akt/Akt ratios in Huh7 and HepG2 hypoxic cells. (**D**) Western blotting results showing the LC3BII/LC3BI ratio in Huh7 and HepG2 hypoxic cells transfected with either Lv-GFP (Ctl), Lv-AQP3 (AQP3+), or Lv-AQP3RNAi (AQP3−). Each experiment was repeated four times, and the mean (SD) is shown in the histogram. Compared with the GFP group (Ctl), the differences were significant as follows: $P < 0.05$; $^{*}P < 0.05$, $^{***}P < 0.001$.

to sorafenib via PI3K/Akt signaling pathway activation. Flow cytometry was used to detect the apoptosis of Huh7 and HepG2 hypoxic cells transfected with Lv-AQP3, Lv-AQP3RNAi, or Lv-GFP, followed by incubation with 6.8μM sorafenib or 6.8μM sorafenib + 50μM LY294002/ 10μM UO126 for 24 h. The results showed a significantly lower apoptosis rate in the Lv-AQP3-transfected group than in the Lv-GFP group (control group). However, the results were reversed in the Lv-AQP3RNAi group (Figure 6A). Co-treatment with LY294002 attenuated the AQP3-induced insensitivity to sorafenib in Lv-AQP3-transfected hypoxic cells. Combined treatment with LY294002 further improved the sorafenib-induced apoptosis of Lv-AQP3RNAi-transfected hypoxic cells (Figure 6B). These results suggest that AQP3 upregulation reduces HCC cell sensitivity to sorafenib by PI3K/Akt signaling pathway activation.

Discussion

In this study, we detected changes in expression of AQP3 in hypoxic HCC cells, and determined whether these changes altered the sensitivity of cells to sorafenib. The results showed that AQP3 is overexpressed in hypoxic HCC cells and altered the sen[illegible] of these cells to sorafenib via PI3K/Akt signal[illegible]g pathway [illegible]ctivation.

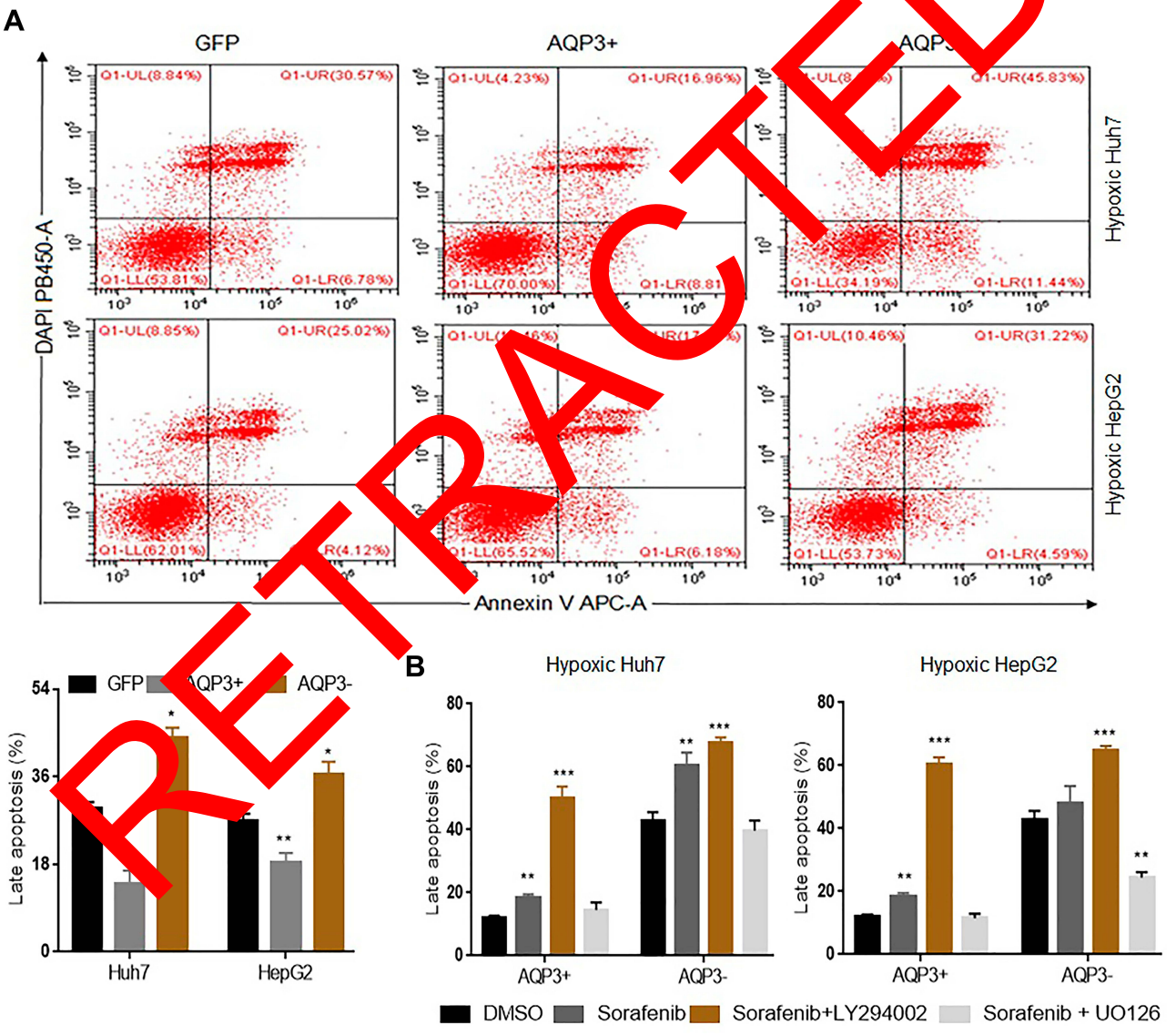

Figure 6 Aquaporin-3 (AQP3) reduces hypoxic hepatocellular carcinoma cell sensitivity to sorafenib by activating the PI3K/Akt signaling pathway. (**A**) Flow cytometry results showing the apoptosis of hypoxic Huh7 and HepG2 cells transfected with either Lv-GFP (Ctl), Lv-AQP3RNAi (AQP3−), or Lv-AQP3 (AQP3+), followed by treatment with 6.8μM sorafenib for 24 h. (**B**) Flow cytometry results showing the apoptosis of hypoxic Huh7 and HepG2 cells transfected with Lv-AQP3 (AQP3+) or Lv-AQP3RNAi (AQP3-) and then treated with either DMSO Ctl), 6.8μM sorafenib alone, 6.8μM sorafenib + 50μM LY294002, or 6.8μM sorafenib + 10μM UO126 for 24 h. Each experiment was repeated four times, and the mean (SD) was shown in a histogram. Compared with the DMSO group (Ctl), the differences were significant as follows: $P < 0.05$; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Intratumoral hypoxia is a hallmark of poor prognosis and treatment response in many solid cancers, including liver cancer.[17,35,38-41] Through changes in gene expression, hypoxia induces, and/or selects for, cells with altered characteristics, including replication potential and stem cell property maintenance, angiogenesis, metabolic reprogramming, metastasis, and radiation and chemotherapy resistance[3,17,35] HIF transcription factors mediate the hypoxia response and activate hypoxia response genes in multiple aspects of tumor development, including aggressiveness and drug resistance.[16–18] Similarly, we showed that hypoxic HCC cells were less sensitive to sorafenib. Although different underlying mechanisms for this phenomenon have been suggested, the results remain controversial and need further clarification.[17,39,41–46]

Studies exploring AQP3's role in the prognosis of various tumors, including HCC, have shown that AQP3 expression is a predictor of poor prognosis.[27,33,47–59] AQP3 is the most overexpressed aquaglyceroporin in HCC.[20–23] In the current study, we found that AQP3 expression was altered in hypoxic HCC cells. AQP3 protein levels increased proportionally to HIF-1α protein levels. Furthermore, hypoxia-activated PI3K/Akt signaling pathway leads to up-regulation of AQP3. Similarly, hypoxia increases the expression of AQP3 mRNA in L929 fibrosarcoma cells.[23] Wang and colleagues also found that AQP3 protein levels correlated with HIF-1α in a matrix-grown Madin–Darby canine kidney cyst model. Therefore, we investigated whether changes in AQP3 expression affected hypoxic HCC cell sensitivity to sorafenib. We found that hypoxic cells transfected with lentivirus expressing AQP3 were less sensitive to sorafenib-induced apoptosis. Additionally, hypoxic cell transfection with Lv-AQP3RNAi restored the sensitivity to sorafenib. These results indicate that AQP3 upregulation is a key hypoxic pro-survival change and contributes to HCC cell adaptation and resistance to sorafenib-induced apoptosis. Our results are consistent with the findings of a study reporting that siRNA inhibition of AQP3 increases prostate cancer cells' susceptibility to cryotherapy.[29]

Furthermore, we investigated the mechanism through which changes in AQP3 expression reduced hypoxic HCC cell sensitivity to sorafenib. Various mechanisms have been reported;[8,13,61–66] however, we focused on the PI3K/Akt and Erk signaling pathways for several reasons, including cross-talk between them that leads to sorafenib resistance,[67] their involvement in AQP3 expression regulation in multiple tumors,[58,68,69] and their involvement in acquired sorafenib resistance in patients with liver cancer.[13,61,62,66] Our results show that both the PI3K/Akt and Erk signaling pathways are downstream effectors of AQP3. The use of LY294002 but not UO126 successfully attenuated the AQP3-induced insensitivity to sorafenib in hypoxic cells transfected with lentivirus overexpressing AQP3. Hypoxic cells with reduced AQP3 levels were more sensitive to sorafenib; this effect was further exacerbated by co-treatment with LY294002. These results showed that the hypoxia-induced PI3K/Akt signaling pathway activation led to AQP3 upregulation, affording a positive feedback regulation on the pathway. Although the exact underlying mechanism is unclear, our findings suggest that the positive feedback by AQP3 upregulation to the PI3K/Akt signaling pathway leads to changes in hypoxic HCC cell sensitivity to sorafenib. These results also suggest a mechanism for further discussion in the literature on how the combined treatment with sorafenib and a PI3K/Akt inhibitor successfully increases HCC cell sensitivity to sorafenib.

Conclusion

Changes in AQP3 expression modulate hypoxic HCC cell sensitivity to sorafenib. The hypoxia-induced AQP3 expression reduces HCC cell sensitivity to sorafenib via PI3K/Akt signaling pathway activation. Therefore, AQP3 is a potential therapeutic target for improving hypoxic HCC cell sensitivity to sorafenib. Further in vivo studies are needed to confirm our findings.

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Disclosure

The authors declare no conflicts of interest regarding this work.

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